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(57) Abstract

A new class of polypeptide molecules, including antibodies, that specifically immunoreact with a cell surface protein that is detectable on human melanoma tumor cells is described, together with specific peptides corresponding to immunoreactive domains of the cell surface protein. Methods of detecting and treatment of human melanoma is further disclosed in the present invention.

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ME20: MONOCLONAL ANTIBODIES AND ANTIGEN FOR HUMAN MELANOMA

TECHNICAL FIELD OF THE INVENTION

The present invention is directed to the discovery of a new cell surface protein designated as antigen ME20 (AgME20) which is present on human melanoma cells and of antibodies, such as mAb ME20 and antibody fragments, which have been developed and specifically immunoreact with this cell surface protein. New peptides that contain regions in their amino acid residue sequences which substantially correspond to domains of AgME20 are also disclosed herein.

BACKGROUND OF THE INVENTION

Tumor cells express certain antigens which are absent from, or present in small amounts on, their normal cellular counterparts. Many of these are differentiation antigens, shared by the tumor and certain embryonic cells. Some of the antigens appear with sufficient selectivity in tumors to serve as possible targets for therapeutic agents. This possibility has been reviewed for malignant melanoma (Hellström and Hellström, in Accomplishment in Cancer Research - 1984 Prize Year, General Motors Cancer Research Foundation, J.G. Fornter & J.E. Rhoads, eds., J.B. Lippincott Company, Philadelphia, (1985) pp. 216-240; Hellström and Hellström, in In Vitro Diagnosis of Human Tumors Using Monoclonal Antibodies, Immunology Series, H.Z. Kupchick, ed. Marcel Dekker, Inc. New York/Basel, (1988) Vol 39, pps. 123-139), as well as for other tumors (Burchell and Taylor-Papadimitrious, in R.W. Baldwin and V.S. Byers, eds., Monoclonal Antibodies for Tumor Detection and Drug Targeting, Academic Press (1985) pps. 1-15; Kemshead, ibid, pp. 281-302).

Many antibodies have been made to cell surface antigens that are expressed in greater quantitites by human tumors than by normal tissues. It has also been well established that some antibodies to cell surface antigens can be cytotoxic to tumor cells in the presence of complement (Hellström et al., (1962) Progr. Allergy 9:158-245; Hellström, and Hellström, in Human Melanoma; S. Ferrone, ed., Springer-Verlag, New York, Berlin, Heidelburg (1990) pps. 442-466), and that some antibodies can mediate antibody-dependent cellular cytotoxicity (Perlmann et al., (1969) Adv. Immunol. 11:117-193; MacLennan et al., (1969), Immunol. 17:896-910; Skurzak et al., (1972) Int. J. Cancer 9:316-323). In the first case, an appropriate source of complement (generally rabbit or guinea pig), and in the latter case a source of effector cells (generally of mouse origin) is needed.

Antibodies such as those directed to tumor-associated antigens can kill human tumor cells in the presence of human effector cells (Hellström et al., (1981) Int. J. Cancer 27:281-285) in the presence of human serum as a source of complement (Hellström et al., (1985) Proc. Natl. Acad. Sci. 82:1499-1502; Hellström et al., (1985) Monoclonal Antibodies and Cancer Therapy, UCLA Symposia on Molecular and Cellular Biology, Vol. 27, pps. 149-164, Alan R. Liss, Inc., NY).

Anti-tumor antibodies have been utilized in immunohistology (Garrigues et al., (1982) Int. J. Cancer 29:511-515; Natali et al., in Carcinogenesis - A Comprehensive Survey, C.J. Conti, T.J. Slaga and A.J.P. Klein-Szanto, eds., Raven Press, Ltd. New York, (1989) Vol. 11. pps. 133-164) and immunotherapy (Hellström and Hellström (1988) Pigment Cell Res. S.1:180-184; Neuwelt et al., (1987) Neurosurgery 20:885-895), as well as for diagnostic imaging (Larson et al., (1983) J. Clin. Invest. 72:2101-2114; Murray et al., (1988) in Malignant Melanoma: Biology, Diagnosis and Therapy, L. Nathanson, ed., Khiwer Academic Publishers, Boston, pps. 123-149).

Some approaches for preparing anti-cancer agents involve labelling antibodies with radioactive isotopes (Larson et al., (1983) Clin. Invest. 72:2101-2114; Order,

(1984) Compr. Ther. 10:9-18; Carrasquillo et al., (1984) Cancer Treatment Reports 68:317-328; de Nardo et al., (1985) Int. J. Radiation Oncology Biol. Phys. 11:335-345), or conjugating antibodies to toxins (Jansen et al., (1982) Immunol. Rev. 62:185-216; Vitetta and Uhr (1984) Transplant. 37:535-538) or anti-cancer drugs, (Ghose et al., (1972) Brit. Med. J. 3:495-499; Hurwitz et al., (1975) Cancer Res. 35:1175-1181; Rowland et al., (1985) Cancer Immunol. Immunother. 19:1-7). In these instances, the antibody provides the specificity of targeting the agent to the appropriate tumor cell and the isotope or drug provides the ability to destroy the tumor. However, a disadvantage of this approach, until the present invention, has been the lack of high specificity of the antibody, which in many instances could bind to non-cancerous tissue as well. Because both anti-cancer drugs and radioisotopes have a high level of toxicity to normal tissues, this nonspecific uptake in various organs such as kidney, liver, or bone-marrow could lead to substantial side-effects.

Another approach toward cancer therapy is to develop specific immunogens, sometimes referred to as therapeutic vaccines (Hellström, K.E., Hellström, I., and Hu, S.-L. (1990) "Why Cancer Vaccines?" In: New Generation Vaccines: The Molecular Approach (Woodrow, G.C., and Levine, M.M., eds.) Marcel Dekker, Inc. pps 855-862). These immunogens are used to induce a state of active tumor immunity in the patients. The level of tumor specificity is crucial for this process, and the present invention addresses this question for melanoma, a highly malignant neoplasm.

SUMMARY OF THE INVENTION

The present invention is directed to a monoclonal antibody, designated mAb ME20, which is immunospecific for a cell surface protein that is found on human melanoma cells and on cells from a fraction of dysplastic nevi but not significantly on other cells. Both melanoma and nevi are believed to be embryologically derived from

the neural crest. This invention also includes fragments of mAb ME20 such as Fab, Fv, Fab' and $(Fab')_2$ fragments as well as other antibodies and fragments with similar specificity. Monoclonal antibody ME20 (and its fragments) can be operatively linked, or conjugated, to another compound such as a drug, toxin, labelling agent, radionuclide, growth factor or enzyme. This invention also includes diagnostic and therapeutic methods employing the antibody as well as methods and compositions for using such monoclonal antibodies.

The present invention is further directed to a novel cell surface antigen, designated AgME20, characteristic of human melanoma cells, as well as a substantially pure peptide that contains a region that corresponds to a domain of a melanoma cell surface antigen such as AgME20. The invention also includes diagnostic and therapeutic methods employing the peptide as well as methods and compositions using that novel peptide.

DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention is directed to compounds and methods useful in the detection and treatment of human melanoma.

Specifically, the present invention is directed to a new monoclonal antibody, mAb ME20 and antibody fragments such as Fab, Fv, Fab' and $F(ab')_2$ fragments, and synthesized polypeptide chains that specifically immunoreact with a human melanoma surface protein, AgME20, to which mAb ME20 binds as described herein. This surface protein is detectably present only on melanoma cells and on cells from a fraction of dysplastic nevi (2 of 6 tested). Both melanoma and nevi are believed to be embryologically derived from neural crest cells. The mAb ME20, its fragments and other similarily binding antibodies and fragments can be utilized, inter alia, in the detection and/or treatment of melanoma either alone or in conjunction with another

compound. In a preferred embodiment, an antibody or fragment of the present invention is operatively linked to a compound such as a drug, toxin, labelling agent, growth factor, radionuclide or radioisotope or an enzyme.

As used herein, the term "immunoreacts" refers to the production of a specific binding interaction similar in nature to the immunological binding that occurs between an antigen and an antibody directed to that antigen. In a preferred embodiment of the present invention, a polypeptide, such as the monoclonal antibody mAb ME20, immunoreacts with a cell surface antigen, such as AgME20, on human melanoma cells. The polypeptides of the present invention bind to a much lesser degree to normal human adult cells and tissue than to melanoma cells.

As used herein, the term "bind to a much lesser degree" means that the binding will not be detectable at all when standard immunohistological techniques are employed. By contrast, mAb ME20 binds with a high degree of specificity to a novel antigen characteristic of human melanoma.

MAb ME20 and similar polypeptides and fragments of the present invention, as described above, can be operatively linked to other compounds and utilized for diagnostic, localization or therapeutic purposes.

As used herein, the terms "polypeptide" and "antibody" are used interchangeably and refer to a natural or synthetic molecule composed of amino acid residues attached by peptide bonds that can specifically immunoreact with a human melanoma cell surface protein, and include glycosylated proteins called immunoglobulins (antibodies), antibody fragments and synthetic proteins and protein fragments that are capable of specifically combining or immunoreacting with a melanoma surface protein.

As used herein, the term "antigen" refers to an entity that is capable of being specifically bound by an antibody. When an antigen is capable of inducing antibody production it is also referred to as an "immunogen".

As used herein the term "operatively linked" refers to a linkage that does not interfere with the ability of either of the linked groups to function as described. In one preferred embodiment, mAb ME20 is operatively linked to a biologically active compound such as a drug in a manner that permits the polypeptide to bind to a human melanoma surface protein and which does not interfere with the biological activity of the drug.

In a particularly preferred embodiment, mAb ME20 is operatively linked to a chemotherapeutic drug, such as dacarbazine, adriamycin or mitomycin C.

The polypeptides of this invention that specifically immunoreact with the ME20 antigen include antibodies such as the monoclonal antibody mAb ME20. The biological activity of antibodies is multi-faceted. To a large extent the Fc region of the molecule determines the ability of antibody molecules to activate complement and mediate antibody-dependent cellular cytotoxicity (ADCC) (Uanue and Benacerraf (1984) Textbook of Immunology, 2nd Edition, Williams and Wilkins, Chap. 12, pps. 218-238).

Antibodies are divided into various classes and subclasses. Preferred monoclonal antibodies of the present invention are of the IgG1, IgG2a, and IgG3 subclasses. In general, antibodies of the IgG2a and IgG3 subclasses can often mediate ADCC and activate serum complement.

The polypeptides of the present invention are preferably present in a pharmaceutical composition together with one or more pharmaceutically acceptable carriers.

As used herein, the term "pharmaceutically acceptable carrier" refers to a compound which is compatible with administration to a patient and does not produce toxic or untoward effects upon such administration. Illustrative examples of pharmaceutically acceptable carriers are phosphate buffered saline, Ringer's solution, oils, gels and microspheres, as well as liposomes. Other pharmaceutically acceptable

carriers are well known in the field of pharmacy and are contemplated by the present invention.

As used herein, the term "labelling agent" refers to a single atom or molecule that when operatively linked to a product of the present invention enables detection of its presence in an assay method. Illustrative labelling agents are fluorescent dyes, radioisotopes, enzymes and antibodies that can be either independently detected or detected in conjuction with the addition of a substrate or other molecule that reacts therewith.

The monoclonal antibody mAb ME20 exists in two isotypic forms, IgG1 and IgG2a, respectively, and these forms have been produced from hybridomas that have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, in full compliance with the deposition requirements of the United States Patent and Trademark Office and the Budapest Treaty, on June 4, 1991 and have the Acccession numbers HB10764 and HB10763 respectively.

The antibodies of the present invention are specifically immunoreactive with determinant sites on a glycoprotein antigen, referred to as AgME20, associated with human melanoma cells and which has not been found on other human tumors. Such immunoreactive specificity for these antibodies greatly diminishes the likelihood of the present antibodies binding to carcinomas of the breast, lung, colon and the like.

It is particularly noteworthy that no binding at all of mAb ME20 to normal human adult cells and tissues has been detected by immunohistological testing.

The antibodies of the present invention are useful, either alone or in combination with other agents, in tumoristatic and/or tumoricidal methods for the treatment of melanoma. These antibodies can be prepared by hybridoma, recombinant or synthetic processes and include fragments, multimers and derivatives thereof. As used herein, the term "multimer" refers to a molecule that contains several repeating units of a portion of the molecule. A multimeric peptide of the present invention

contains, preferably, 2 to about 10 repeating regions of a portion of an amino acid residue sequence of the peptide operatively linked together. In one embodiment of the present invention a multimeric antibody is contemplated that corresponds to several molecules of an antibody, such as mAb ME20, operatively linked together to result in a molecule having several functional binding sites that can each specifically immunoreact with a human melanoma cell surface protein such as AgME20. The antibodies of this invention can be further utilized in methods and tests for diagnosis, detection and treatment of human melanoma.

The present invention further encompasses a substantially purified peptide that contains a region which mimics, or substantially corresponds to, a domain on a human melanoma cell surface protein. The peptides of the present invention are capable of specifically immunoreacting with an antibody of the present invention, such as mAb ME20.

As used herein the term "substantially" refers to a high level of similarity. A substantially purified peptide of the present invention refers to a preparation having less than about ten percent extraneous peptides present. A substantially similar sequence in the present invention has less than about ten percent variation with the reference sequence.

In a preferred embodiment, a peptide of the present invention include substantially purified natural and synthetic molecules that contain amino acid residue sequences and/or glycosylation that can immunoreact with the ME20 monoclonal antibody.

These peptides can be prepared by standard methods of protein purification, and by either chemical or recombinant synthesis, and include fragments, multimers and derivatives thereof that retain the ability to specifically immunoreact with the antibodies of the present invention.

The ME20 human melanoma cell surface protein, AgME20, is a glycoprotein that has a molecular weight of about 80 kD to about 120 kD. In a preferred embodiment, the ME20 cell surface protein has a molecular weight, as determined by SDS-PAGE, of about 100 kD to about 116 kD.

The antibodies of the present invention can be utilized in methods of treatment and for detection of human melanoma.

In one embodiment, an antibody of the present invention that is capable of binding to a human melanoma cell surface protein can be utilized to detect the presence of human melanoma cells in in vitro and in vivo assays. In this method human cells of a patient are contacted with an effective amount of the antibody to enable binding of the antibody to any melanoma cells that are present. The presence of the bound antibody is then detected by any of several methods that are readily available in the art. Illustrative detection methods include radiographic detection of the presence of a radioisotope operatively linked to the antibody, use of a second antibody that is detectably labelled and which second antibody immunoreacts with an exposed region of the bound antibody, and the use of an indicating means, such as a substrate which is converted to a fluorescent molecule by an enzyme operatively linked to the antibody.

In another embodiment, a polypeptide of the present invention, such as mAb ME20, is administered at a therapeutically effective dosage to a patient together with a chemotherapeutic agent for a time period that is sufficient to result in the inhibition of further proliferation of any melanoma cells present. Illustrative chemotherapeutic agents are preferrably drugs and toxins commonly utilized in the treatment of melanoma. The chemotherapeutic agent can be added separately from the polypeptide of this invention, together with the polypeptide or operatively linked to the polypeptide, such as a polypeptide-drug conjugate.

The present invention also encompasses an immunogenic composition capable of engendering an immune response when administered to a patient. These immunogenic

compositions contain a substantially purified peptide of the present invention, corresponding to a region of a human melanoma cell surface protein, and an immune response enhancing agent such as a hapten or adjuvant, such as alum. This cell surface protein, such as AgME20, may also be presented to a patient in various types of "vaccines", for example, AgME20 may be expressed in a recombinant vaccinia virus (Estin et al., (1988) Proc. Natl. Acad. Sci. USA 85:1052-1056, containing the gene encoding the 80 - 120 kD melanoma-associated peptide or part thereof. Other specific immunogens relating to AgME20, such as anti-idiotypic antibodies, are also claimed as part of this invention.

In one embodiment of the present invention, an immunogenic composition containing a peptide of the present invention is administered to a patient in a sufficient dosage to elicit an immune response directed toward human melanoma cells. It is contemplated by the present invention that the immunogenic peptide composition can engender an immune response in a patient that is specifically directed against an epitope present on a human melanoma cell surface protein such as AgME20.

Kits containing antibodies and/or peptides of the present invention are also contemplated, wherein these kits further contain appropriate instructions for use of the kit. In a specific embodiment, the kit of the present invention contains the monoclonal antibody ME20.

The present invention is further described by the following Examples which are intended to be illustrative and not limiting.

EXAMPLE 1

ME20 MONOCLONAL ANTIBODY

A. Preparation of ME20 Antibody

Balb/C mice (approximately 2 months old) received six innoculations, both subcutaneous (sc) and intraperitoneal (ip), with human melanoma cells. The first four innoculations were approximately one-month apart, the fifth innoculation was one week later and the sixth was three weeks after the fifth inoculations. Each innoculation contained approximately 10^7 cells of H3614 and/or H3606 human melanoma cells (obtained from primary tumors); innoculations 1, 2 and 3 contained H3614 cells, innocuation 4, 5 and 6 contained both H3614 and H3606 cells.

Three days following the sixth innoculation, the spleen was removed from a mouse and used in the fusion protocol. The spleen was disrupted into cells by scraping through screens and rinsed with RPMI medium. The spleen cells were separated from the remaining tissue by centrifugation at 200 x g for 10 minutes, resuspended in RPMI medium and then mixed with Ag8-653 myeloma cells in a ratio of Ag8-653/spleen cells of about 1:10.

The mixture of Ag8-653 and immunized spleen cells was centrifuged at 200 x g for 10 minutes, the supernatant medium was removed and the cells were maintained at 37°C.

Polyethylene glycol (PEG, 1 ml) was added to the cells over a period of one minute with shaking for an additional one minute.

Iscove's Modified Dulbecco's Medium (IMDM) without HAT (hypoxanthine, aminopterin, thymidine) was added (1 ml over a period of 1 minute); followed by an additional 8 ml over 2 minutes. The cells were centrifuged for 10 minutes at 160 x g at room temperture. The supernatant was removed and the cells were resupsended in 25 ml of IMDM. The resuspended cells were then mixed with 2 x 10^8 mouse thymocytes obtained fresh from 3 - 4 week old Balb/C mice.

The mixed cell culture of Ag8-653 cells, spleen cells and thymocytes was supsended in a volume of 98 ml of IMDM medium and 2 ml of 50 x HAT. The culture was maintained at 37°C/7% CO_2 for 12 to 16 hours, and the cells were then

transferred to 96-well microtiter plates at 200 μl/well and maintained at 37°C/7% CO_2. After 3 days, the plates were microscopically examined to determine fusion efficiency.

The hybrid cells were then screened for antibody production and positive hybrids were transferred into 48-well plates with additional thymocytes.

The resultant antibodies produced by the hybrid clones were then screened by enzyme-linked immunosorbent assay (ELISA) upon the immunizing melanoma cell lines and fibroblasts (human skin fibroblasts). The ME20 antibody was isolated based upon its property of binding to melanoma cells but not binding to fibroblasts. Further characterization of mAb ME20 was obtained by immunohistology and cell analysis on FACS.

Studies were performed using fluorescent-activated cell sorting (FACS) at both 4°C and 37°C, on H3606 melanoma cells. The results, described below, showed that the ME20 antibody bound primarily to the melanoma cell surface at 4°C and at 37°C the antibody primarily was detected in the perinuclear region. The perinuclear localization was further confirmed by Confocal microscopy. The antibody internalizes at 37°C.

B. Immunohistology

The binding of mAb ME20 to various normal and tumor human tissues was monitored by the peroxidase-anti-peroxidase (PAP) immunohistological techniques described in Hellström et al., (1983) J. Immunol. 130:1467. The results shown in TABLE 1 demonstrate that the mAb ME20 specifically binds to human melanoma and binds to an approximately 1/3 of dysplastic nevi assayed, but does not bind to normal human tissue. Both melanoma and nevi cells are believed to be embryologically derived from the neural crest and may share or express some common surface antigens.

The lack of any detectable binding of mAb ME20 to normal tissue demonstrates that this antibody is highly specific and useful for the detection and treatment of human melanoma.

TABLE 1

IMMUNOHISTOLOGY

TUMOR TISSUE			NORMAL TISSUE		
Type	Positive/No. Tested		Type	Positive/No. Tested	
Breast	0	10	Adrenal	0	3
Colon	0	10	Bowel	0	1
Lung	0	15	Brain	0	1
Melanoma	18	19	Breast	0	3
Ovary	0	2	Colon	0	6
Nevi (dysplastic)	2	6	Esophagus	0	3
			Heart	0	4
			Kidney	0	10
			Liver	0	8
			Lung	0	8
			Lymph node	0	4
			Optic Nerve	0	1
			Ovary	0	2
			Pancreas	0	6
			Skin	0	5
			Spleen	0	9
			Stomach	0	4
			Testis	0	2
			Thyroid	0	2
			Tonsil	0	1

C. Characterization Of ME20 Antigen With mAb ME20

H3606 melanoma cells were metabolically labelled with ^{35}S-methionine for 10 min followed by incubation in methionine-free RPMI-1640 medium, supplemented with 5% dialyzed fetal bovine serum, for 0 to 10 hr at 37°C. The cell pellet was extracted with phosphate-buffered saline, pH 7.4, containing either 1% NP-40, PMSF (10

μg/ml) and aprotinin (20 μg/ml) or 0.4% Triton X-100 and proteinase inhibitors. ME20 antigen was immunoprecipitated by incubating the cell lysate with mAb ME20 for 15 min at 4°C. The antigen-antibody complex was precipitated with rabbit anti-mouse IgG and Protein A-Sepharose (Sigma Chemical Co., St. Louis, MO). The washed immunoprecipitate was analyzed by SDS-PAGE under reducing conditions and visualized by fluorography after impregnating the gel with AMPLIFY (Amersham, Arlington Heights, IL).

H3606 human melanoma cells were metabolically labelled with ^{3}H-glucosamine by incubation in 50% ISCOVE's DMEM, 50% glucose-free RPMI-1640, and 5% dialyzed fetal bovine serum. ME20 antigen was immunoprecipitated from the cell lysate with mAb ME20, rabbit anti-mouse IgG, and Protein A-Sepharose. The immunoprecipitate was analyzed by SDS-PAGE under reducing conditions and visualized by autoradiography.

A major Mr = 105,000 band (as determined by SDS-PAGE on 7.5% polyacrylamide gels) of AgME20 appeared within 10 min of labelling and remained the predominant band during chase up to 1 h, but became very weak by 3 h. A high molecular weight component of Mr = 120,000 appeared at 10 min chase and was present for 2 h. By longer chase times (4 to 10 h) a Mr = 97,000 band was the most predominant component immunoprecipitated by mAb ME20.

ME20 antibody specifically precipitated glycoprotein antigens with Mr = 120,000, Mr = 105,00, and Mr = 97,000 as determined by SDS-PAGE on 7.5% polyacrylamide gels. The data demonstrate that the antigenic determinant recognized by mAb ME20 antibody is localized on a novel glycoprotein. The membrane-bound antigen is released into the conditioned medium of H3606 cells. The soluble form of AgME20 has a Mr = 97,000. Aminoterminal sequence analysis indicate that the soluble form of AgME20 has the same aminoterminal sequence as the membrane-bound ME20 antigen and is also a single-chain glycoprotein.

D. Characterization of Monoclonal Antibody ME20 Binding

The ability of mAb ME20 to bind and internalize into tumor cells was investigated by an indirect immunotoxin method to detect the internalization of an antibody-toxin conjugate with subsequent killing of cells that internalize the conjugate, according to the method of Till et al., (1988) Cancer Res. 48:1119-1123. A Fab fragment of goat anti-mouse immunoglobulin coupled to ricin A chain was reacted with cells previously exposed to 10 μg/ml of either mAb ME20 or mAb BR96. MAb BR96 is a monoclonal antibody that specifically binds to breast, colon and lung cancer cells and is internalized by BR96 antigen-positive cells (Hellström et al., (1990) Cancer Res. 50:2183-2190).

The percent inhibition of survival of the cells tested together with the binding ratio, as measured by FACS, is shown in TABLE 2, and demonstrates the internalization of mAb into human melanoma cells, as evidenced by the specific killing of melanoma cells for mAb ME20.

TABLE 2

Cell Line	Antibody	% Inhibition	FACS Binding Ratio
3606 (melanoma)	ME20	95	16
	BR96[1]	26	1
3774 (melanoma)	ME20	82	2
	BR96	8	1
2669 (melanoma)	ME20	94	5
	BR96	5	1
HT29 (colon)	ME20	0	1
	BR96	6	1
2707 (lung)	ME20	31	1
	BR96	98	29
3396 (breast)	ME20	17	1
	BR96	99	54

1 MAb BR96 binds to breast, colon, and lung cancer cells and is internalized by antigen positive cells.

The FACS analysis of mAb ME20 binding, as shown in TABLE 2, measured the binding of a fluorescein isothiocynate (FITC)-labelled secondary antibody to tumor cells in vitro that had been treated with 10 μg/ml of either mAb BR96 or mAb ME20.

Further studies were carried out, as shown in TABLE 3, utilizing a FITC labelled secondary goat anti-mouse antibody.

The binding ratio represents a ratio between the brightness, designated linear fluorescence equivalent (LFE), of cells stained by the specific mAb ME20 versus the unstained cell population.

$$\frac{LFE_{sample}}{LFE_{control}} = \text{Binding Ratio}$$

TABLE 3

Cell Line	Type	FITC Binding Ratio
3606	Melanoma	16
2669	Melanoma	5
3650	Melanoma	2
3620	Melanoma	2
3614	Melanoma	1
3677	Melanoma	1
3720	Melanoma	1
3721	Melanoma	1
3774	Melanoma	2
3630	Breast	1
3396	Breast	1
MCF-7	Breast	1
HT29	Colon	1
RCA	Colon	1
2707	Lung	1
2981	Lung	1
B-Cell Lymphoma	PBL	1
T-Cell Lymphoma	PBL	1

Confocal microscopy was carried out on H3606 human melanoma cells in order to further illustrate the internalization of mAb ME20. Viable H3606 cells were stained by exposure at 4°C to mAb ME20 conjugated to phycoerythrin (mAb ME20-PE). The labelled antibody associated with microvilli located on the plasma membrane of the cells.

When similarly labelled cells were then maintained at 37°C for 2 hours the mAb ME20-PE had a diffuse cytoplasmic perinuclear localization and was largely absent from the cell surface indicating that the antibody had been internalized.

These studies indicated the apparent native distribution of AgME20. Cells which had been previously detergent permeabilized and fixed with paraformaldehyde were exposed to mAb ME20-PE. Cell surface distribution of antibody binding was seen similar to that observed above for mAb ME20 binding at 4°C. Additionally,

intense staining was also associated with an acentric perinuclear compartment, possibly representing the Golgi apparatus. These observations suggest that newly synthesized AgME20 is transported through the Golgi apparatus, exported to the plasma membrane surface and is then internalized following binding by mAb ME20.

The avidity of mAb ME20 binding to H3606 human melanoma cells was studied for two isotypes of mAb ME20. Iodinated mAb ME20 (^{125}I-mAbME20) was assayed for immunoreactivity to antigen positive cells and then utilized in binding studies on H3606 cells. Scatchard analysis was carried out and the results shown a Ka of approximately 10^{-8}M for both the IgG1 and IgG2a isotypes of mAb ME20.

EXAMPLE 2

ME20 ANTIGEN

A. Purification

ME20 antigen (AgME20) was isolated from human melanoma tumor cells (H3606 cells) obtained from a metastatic lesion established as a cell line by the inventors and partially purified by immunoaffinity chromatography. H3606 cells were suspended in phosphate-buffered saline (PBS). An equal volume of solubilization buffer was added to reach a final concentration of 0.4 M NaCl, 1% Triton X-100, 10 mM EDTA in 17 mM phosphate buffer, pH 7.4. The buffer contained the following proteinase inhibitors, 1 mM PMSF (phenylmethylsulfonyl flouride), 2 mM BAEE (Nα-benzoyl-L-arginine ethyl ester), 1 μg/ml each of leupeptin, aprotinin, and pepstatin. The cells were treated with solubilization buffer by gentle agitation for 30 min, at 4°C. Insoluble material was removed by centrifugation at 100,000 x g for 90 min, at 4°C. ME20 antigen was purified from the supernatant by immunoaffinity chromatography.

MAb ME20 mAb (5 mg/ml) in 0.2 M sodium phosphate buffer containing 0.5 M NaCl, pH 8.2, was added to 1 g of wet tresyl-Sepharose (Sigma). Coupling continued with gentle agitation by tumbling for 16 h, at 4°C. The gel was treated with 0.2 M Tris-HCl, pH 8.5, for 5 h, at 20°C, and washed with: (1) 0.2 M sodium acetate buffer containing 0.5 M NaCl, pH 3.5; (2) 17 mM phosphate buffer containing 0.4 M NaCl and 1% Triton X-100, pH 7.4; (3) phosphate-buffered saline containing 1% Triton X-100, pH 7.4; (4) 100 mM diethylamine containing 0.2% Triton X-100, pH 11.5; and (5) phosphate-buffered saline containing 1% Triton X-100.

The cell lysate was applied to a 1 ml column of (mAb ME20)-Sepharose and recirculated for 12 to 16 hours, at 4°C. The affinity support was washed with PBS containing 0.2% Triton X-100 and the antigen was eluted with 100 mM diethylamine containing 0.2% Triton X-100, pH 11.5. The eluate was neutralized with 2M Tris-HCl buffer, pH 6.8.

The partially purified AgME20 was futher purified by SDS-PAGE. The column eluate was dialyzed against 0.1% SDS. SDS-PAGE (10% acrylamide) was performed according to the Laemmli method using minislab gels with 0.75 mm-thick spacers (BioRad).

Following electrophoresis, the SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue and then destained. The stained ME20 antigen bands (Mr = 116,000 and 100,000) were excised with a razor blade and subjected to electroelution.

ME20 antigen was also recovered from SDS-polyacrylamide gels by electroblotting onto Problot membrane (Applied Biosystems, Inc.) using Mini-Transblot Electrophoretic Transfer Cell (BioRad Laboratories, Richmond, CA), as described (Matsudaira, P., (1987), J. Biol. Chem. 261:10035-10038). The membrane was dyed with Coomassie Brilliant Blue, destained, and the stained AgME20 bands (Mr = 116,000 and 100,000) were excised with a razor blade for subsequent aminoterminal sequence analysis.

B. Sequence Analysis

Automated Edman degradation was performed on three preparations with 9.9 pmol, 7.7 pmol, and 3.0 pmol, respectively, each of AgME20 in a pulsed-liquid protein sequencer, model 475A (Applied Biosystems, Inc.) equipped with a vertical cross-flow reaction cartridge, as described by Hewick et al. (Hewick, R.M., et al. (1981) J. Biol. Chem. 256:7990-7997).

The phenylthiohydantoin (PTH) amino acid derivatives were analyzed by reverse phase high performance liquid chromatography (rpHPLC) using a model 120A on-line HPLC unit (Applied Biosystems, Inc.) with PTH C18 column (2.1 X 220 mm, Applied Biosystems, Inc.) and a sodium acetate/tetrahydrofuran/acetonitrile gradient for elution. Data reduction and quantitation were performed by using a Nelson 760 interface, a Hewlett Packard 9816 computer, and model 900A/model 475A chromatogram analysis software (Applied Biosystems, Inc.)

Sequence studies indicated that 3 major membrane-bound ME20 antigens (Mr = 116,000; 100,000; and 80,000, as determined by SDS-PAGE on 10% polyacrylamide gels) have identical amino-terminal amino acid sequences. The aminoterminal sequence, designated as Sequence I.D. No. 1, is as follows:

```
K   V   P   R   N   Q   D   W   L   G
Lys Val Pro Arg Asn Gln Asp Trp Leu Gly
1               5                   10

V   S   R   Q   L   R   T   K   A   W
Val Ser Arg Gln Leu Arg Thr Lys Ala Trp
                15                  20

N   R   Q   L   Y   P   E   W   T   X
Asn Arg Gln Leu Tyr Pro Glu Trp Thr X
                25
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(X: the amino acid has not been identified.)

In addition, a soluble form of AgME20 was isolated from the conditioned medium of H3606 cells. The antigen was purified by affinity chromatography and recovered from the mAb ME20 column by elution with 100 mM diethylamine, pH 11.5, in the absence of Triton X-100. The Mr of the soluble antigen was 97,000.

The aminoterminal sequence of AgME20 antigen was compared against the most updated PIR database (release 27) and the MIPSX merged database. The sequence comparison did not reveal significant matches with any other known sequence.

The foregoing description and Examples are intended as illustrative of the present invention, but not as limiting. Numerous variations and modifications may be effected without departing from the true spirit and scope of the present invention.

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 Amino acids
(B) TYPE: Amino acid
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens
(F) TISSUE TYPE: Melanoma
(H) CELL LINE: H3606 Human Melanoma Cells

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Lys Val Pro Arg Asn Gln Asp Trp Leu Gly
1               5                  10

Val Ser Arg Gln Leu Arg Thr Lys Ala Trp
                15                  20

Asn Arg Gln Leu Tyr Pro Glu Trp Thr
                25
```

We claim:

1. An antibody that specifically immunoreacts with a human melanoma cell surface protein, said surface protein having a molecular weight of about 80 to about 120 kD and characterized as being present on human melanoma tumor cells but not on other human tumor cells.

2. The antibody according to Claim 1, wherein the antibody has the binding characteristics of monoclonal antibody ME20, having an IgG1 isotype as produced by a hybridoma having ATCC Accession Number HB10764.

3. The antibody according to Claim 1, wherein the antibody has the binding characteristics of monoclonal antibody ME20, having an IgG2a isotype as produced by a hybridoma having ATCC Accession Number HB10763.

4. The antibody according to Claim 1, wherein the cell surface protein has a molecular weight of about 100 kDa.

5. The antibody according to Claim 1, wherein the cell surface protein is a glycosylated protein having an aminoterminal amino acid residue sequence substantially corresponding to Sequence I.D. No. 1.

6. The antibody according to Claim 5, wherein the surface protein has a molecular weight of about 116 kDa.

7. The antibody according to Claim 1, wherein the polypeptide is an antibody fragment capable of specifically immunoreacting with the melanoma cell surface protein.

8. The antibody according to Claim 7, wherein the antibody fragment comprises an Fab, Fv, Fab' or $F(ab')_2$ fragment.

9. The antibody according to Claim 1, wherein the antibody further comprises a compound operatively linked to the antibody.

10. The antibody according to Claim 9, wherein the compound is selected from the group consisting of a labelling agent, a drug, a toxin, a growth factor, a radionuclide and an enzyme.

11. The antibody according to Claim 10, wherein said drug is dacarbazine.

12. An antibody fragment that is capable of specifically immunoreacting with a human melanoma cell surface protein, said cell surface protein having a molecular weight of about 80 kDa to about 120 kDa.

13. The antibody fragment according to Claim 12, wherein the fragment comprises an Fab, Fv, Fab' or $(Fab')_2$ fragment.

14. A pharmaceutical composition comprising an antibody that specifically immunoreacts with a human melanoma cell surface protein, together with a pharmaceutically acceptable carrier.

15. The composition according to Claim 14, wherein the antibody is a monoclonal antibody having the binding characteristics of monoclonal antibody ME20

having an IgG1 isotype, that is produced by a hybridoma having ATCC Accession Number HB10764.

16. The composition according to Claim 14, wherein the antibody is a monoclonal antibody having the binding characteristics of monoclonal antibody ME20, having an IgG2a isotype, that is produced by a hybridoma having ATCC Accession Number HB10763

17. The composition according to Claim 14, wherein the antibody is an antibody fragment.

18. The composition according to Claim 17, wherein the antibody fragment comprises an Fab, Fv, Fab' or $(Fab')_2$ fragment.

19. A method of determining the presence of human melanoma cells comprising

(a) contacting human cells with an effective amount of a antibody capable of specifically immunoreacting with a human melanoma cell surface protein to enable binding of said antibody to any human melanoma cells present, and

(b) detecting the binding of the antibody to the melanoma cell surface protein.

20. The method according to Claim 19, wherein said antibody is an antibody, or fragment thereof, having binding characteristics of monoclonal antibody ME20, having an IgG1 isotype that is produced by a hybridoma having the ATCC Accession Number HB10764.

21. The method according to Claim 19, wherein said antibody is an antibody, or fragment thereof, having binding characteristics of monoclonal antibody ME20, having an IgG2a isotype that is produced by a hybridoma having the ATCC Accession Number HB10763.

22. The method according to Claim 19, wherein the binding of the antibody to human melanoma cells is determined by detecting the presence of a labelling means operatively linked to the antibody.

23. The method according to Claim 22, wherein the labelling means is a radionuclide.

24. The method according to Claim 22, wherein the labelling means is a fluorescent label.

25. The method according to Claim 22, wherein the labelling means is an enzyme.

26. The method according to Claim 19, wherein the cells and the antibody are contacted in vitro.

27. The method according to Claim 21, wherein the cells and the antibody are contacted in vivo.

28. A method for locating melanoma cells in a patient comprising administering to the patient an effective amount of an antibody capable of specifically immunoreacting with a cell surface protein on human melanoma cells for a time

period sufficient for the antibody to specifically bind to the melanoma cells, and then detecting the presence of any melanoma cells with the antibody bound thereto.

29. The method according to Claim 28, wherein the presence of the melanoma cells is determined by detecting the presence of a labelling means operatively linked to the antibody.

30. The method according to Claim 28, wherein the presence of the melanoma cells is determined by administering a reagent to the patient that is capable of binding to and indicating the presence of antibody bound melanoma cells.

31. The method according to Claim 30, wherein the reagent is a second antibody capable of binding to melanoma-bound antibody, said second antibody being operatively linked to an indicating means.

32. The method according to Claim 29, wherein the labelling means is a radioisotope.

33. A method of treating melanoma in a patient comprising administering to the patient a therapeutically effective amount of an antibody capable of specifically immunoreacting with a cell surface protein on human melanoma cells together with a chemotherapeutic agent for a time period sufficient to inhibit the proliferation of the melanoma cells.

34. The method according to Claim 33 wherein the antibody is operatively linked to a compound selected from the group consisting of a drug, a toxin, a growth factor, a radionuclide and an enzyme.

35. The method according to Claim 33, wherein the chemotherapeutic agent is a drug selected from the group consisting of doxorubicin, dacarbazine mitomycin C, cis-platin and a nitrosourea.

36. The method according to Claim 34, wherein the compound is a radioisotope.

37. A substantially purified peptide that contains a region which substantially corresponds to a domain of a human melanoma cell surface protein or fragment thereof, said region being capable of specifically immunoreacting with monoclonal antibody ME20 as produced by the hybridoma having ATCC Accession Number HB10764 or ATCC Accession Number HB10763.

38. The peptide according to Claim 37, wherein the peptide is operatively linked to a compound selected from the group consisting of a labelling agent, a drug, a toxin, a growth factor, a radionuclide, a hapten and an enzyme.

39. The peptide according to Claim 37, wherein said melanoma cell surface protein has a molecular weight of about 80 kD to about 116 kD, and an aminoterminal amino acid residue sequence substantially corresponding to Sequence I.D. No. 1.

40. The peptide according to Claim 39, wherein the peptide contains a region substantially corresponding to a glycosylated domain of a 100 kD protein.

41. The peptide according to Claim 39, wherein the peptide contains a region substantially corresponding to a glycosylated domain of a 116 kD protein.

42. The peptide according to Claim 37 wherein the peptide is produced by recombinant expression.

43. A pharmaceutical composition comprising a substantially purified peptide that contains a region which substantially corresponds to a domain of a human melanoma surface protein or fragment thereof, said region being capable of specifically immunoreacting with monoclonal antibody ME20 as produced by the hybridoma having ATCC Accession Number HB10764 or ATCC Accession Number HB10763 and a physiologically tolerable carrier.

44. An immunogenic composition comprising a substantially purified peptide that contains a region which substantially corresponds to a domain of a human melanoma cell surface protein or fragment thereof, said region being capable of immunoreacting with an antibody having binding characteristics of monoclonal antibody ME20 as produced by a hybridoma having ATCC Accession Number HB10764 or ATCC Accession Number HB10763 and an immune responding enhancing agent.

45. The composition according to Claim 44, wherein the immune response enhancing agent is operatively linked to the peptide.

46. The composition according to Claim 44, wherein the immune response enhancing agent is a hapten.

47. The composition according to Claim 44, wherein the immune response enhancing agent is a physiologically tolerable adjuvant.

48. The composition according to Claim 47, wherein the adjuvant is alum.

49. A method of inducing an immune response in a patient directed toward human melanoma comprising administering to the patient an immunogenic amount of a peptide that contains a region which substantially corresponds to a domain of a human melanoma surface protein, or a fragment thereof, said region being capable of immunoreacting with an antibody having binding characteristics of monoclonal antibody ME20 as produced by a hybridoma having ATCC Accession Number HB10764 or ATCC Accession Number HB10763.

50. The method according to Claim 49, further comprising administering an immune response enhancing agent to the patient.

51. A kit comprising:

(a) a package containing an antibody, or fragment thereof, that specifically immunoreacts with a human melanoma cell surface protein, and

(b) instructions for use.

52. The kit according to Claim 53, wherein the antibody has the binding characteristics of the monoclonal antibody ME20 produced by a hybridoma having ATCC Accession Number HB10764 or ATCC Accession Number HB10763.

53. The kit according to Claim 51, further comprising a package containing, a substantially purified peptide that contains a region which substantially corresponds to a domain of a human melanoma cell surface protein, or fragment thereof, said region being capable of specifically immunoreacting with monoclonal antibody ME20 as produced by the hybridoma having ATCC Accession Number HB10763 or ATCC Accession Number HB10763.

54. A kit comprising:

(a) a package containing a substantially purified peptide that contains a region which substantially corresponds to a domain of a human melanoma cell surface protein, or fragment thereof, said region being capable of specifically immunoreacting with monoclonal antibody ME20 as produced by the hybridoma having ATCC Accession Number HB10764 or ATCC Accession Number HB10763 and;

(b) instructions for use.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/04451

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12P21/08; A61K39/395; G01N33/574; G01N33/577
A61K47/48; A61K49/00; C07K15/14; A61K39/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12P ; C07K ; A61K ; G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	MOLECULAR IMMUNOLOGY vol. 18, no. 3, March 1981, OXFORD, GB pages 207 - 218; K. MITCHELL ET AL.: 'Structural characterization of the "melanoma-specific" antigen detected by monoclonal antibody 691I5Nu-4-B.' see abstract	1-4,7,8, 12,13, 19-21,26
X	JOURNAL OF NUCLEAR MEDICINE vol. 26, no. 10, October 1985, NEW YORK, US pages 1172 - 1179; P. BEAUMIER ET AL.: 'Melanoma localization in nude mice with monoclonal Fab against p97.' see the whole document	1,4, 7-10, 12-14, 17-19, 22,23, 26-29, 32,51 11
	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 SEPTEMBER 1992	- 2. 10. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	NOOIJ F.J.M.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/04451

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 19 - 25 (partially, as far as an in vivo diganostic metho
concerned) and claims 27 - 32 are directed to a diagnostic method practised on the
human/animal body, the search has been carried out and based on the alleged effect
of the compound/composition.

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2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/

Remark: Although claims 33 - 36, 49 and 50 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9204451 SA 61099

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/09/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0189849	06-08-86	JP-A-	62289524	16-12-87
GB-A-2188637	07-10-87	BE-A-	1000175	12-07-88
		CH-A-	675424	28-09-90
		DE-A-	3703702	18-02-88
		NL-A-	8700285	01-09-87
		OA-A-	8478	29-07-88
		SE-A-	8700466	08-08-87
		AU-B-	606046	31-01-91
		AU-A-	6862087	13-08-87
		FR-A-	2601370	15-01-88
		JP-A-	62294698	22-12-87
		LU-A-	86765	15-09-87

FORM P0479